The Plant Cell, Vol. 6, 645–657, May 1994 © 1994 American Society of Plant Physiologists

Soybean *GH3* Promoter Contains Multiple Auxin-Inducible Elements

Zhan-Bin Liu, Tim Ulmasov, Xiangyang Shi, Gretchen Hagen, and Tom J. Guilfoyle[1]

Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, Missouri 65211

The soybean *GH3* gene is transcriptionally induced in a wide variety of tissues and organs within minutes after auxin application. To determine the sequence elements that confer auxin inducibility to the *GH3* promoter, we used gel mobility shift assays, methylation interference, deletion analysis, linker scanning, site-directed mutagenesis, and gain-of-function analysis with a minimal cauliflower mosaic virus 35S promoter. We identified at least three sequence elements within the *GH3* promoter that are auxin inducible and can function independently of one another. Two of these elements are found in a 76-bp fragment, and these consist of two independent 25- and 32-bp auxin-inducible elements. Both of these 25- and 32-bp auxin-inducible elements contain the sequence TGTCTC just upstream of an AATAAG. An additional auxin-inducible element was found upstream of the 76-bp auxin-inducible fragment; this can function independently of the 76-bp fragment. Two TGA-box or Hex-like elements (TGACGTAA and TGACGTGGC) in the promoter, which are strong binding sites for proteins in plant nuclear extracts, may also elevate the level of auxin inducibility of the *GH3* promoter. The multiple auxin-inducible elements within the *GH3* promoter contribute incrementally to the overall level of auxin induction observed with this promoter.

INTRODUCTION

A number of auxin-regulated genes and cDNAs have been identified in plants. These include genes and/or cDNAs from *Nicotiana* suspension-cultured cells (Takahashi et al., 1989, 1991; van der Zaal et al., 1991; Dominov et al., 1992; Ishida et al., 1993), soybean hypocotyl (Walker and Key, 1982; Hagen et al., 1984; McClure and Guilfoyle, 1987), pea epicotyl (Theologis et al., 1985; B.A. McClure, G. Hagen, and T.J. Guilfoyle, unpublished results), mung bean epicotyl (Yamamoto et al., 1992a, 1992b), and Arabidopsis seedlings (Alliotte et al., 1989; Conner et al., 1990; G. Hagen and T.J. Guilfoyle, unpublished results). In addition, a few genes encoded on the *Agrobacterium tumefaciens* and *A. rhizogenes* T-DNAs have been reported to be regulated by auxin (An et al., 1990; Maurel et al., 1990; Korber et al., 1991; Kim et al., 1994). In some cases, the genes appear to be specifically responsive to applied auxins, but in other cases, a number of additional agents (e.g., cytokinin, salicylic acid, methyl jasmonate, heavy metals, and a variety of other chemicals), wounding, and environmental stress induce gene expression (reviewed by Guilfoyle et al., 1993).

We have cloned a gene from soybean, the *GH3* gene, that is transcriptionally induced within 5 min after exogenous auxin application to soybean plumules (Hagen and Guilfoyle, 1985). The inducibility of the *GH3* promoter is specific to auxins (Hagen and Guilfoyle, 1985; Hagen et al., 1991). We have previously described the expression patterns of the soybean *GH3* promoter (592 bp in length) fused to the β-glucuronidase (*GUS*) reporter gene in transgenic tobacco plants (Hagen et al., 1991). Our results indicated that this promoter fusion gene was responsive to exogenous auxin in most, if not all, organs and tissues of the transformed tobacco plants at various stages in the plant life cycle.

A number of putative auxin-responsive elements (AuxREs) have been reported (reviewed by Guilfoyle, 1994). Some of these putative AuxREs have been identified strictly by sequence comparisons among a number of auxin-regulated genes (Ainley et al., 1988; McClure et al., 1989; Conner et al., 1990; Hagen et al., 1991; Oeller et al., 1993), whereas others have been identified by promoter deletion analysis, gain-of-function analysis with a minimal cauliflower mosaic virus (CaMV) 35S promoter, DNase I footprinting, and sequence comparisons (An et al., 1990; Korber et al., 1991; Ballas et al., 1993; Nagao et al., 1993; Kim et al., 1994; Liu and Lam, 1994; T. Ulmasov, A. Ohmiya, G. Hagen, and T.J. Guilfoyle, manuscript in preparation; Y. Li, Z.-B. Liu, X. Shi, G. Hagen, and T.J. Guilfoyle, manuscript submitted for publication).

The bulk of these putative AuxREs in different genes bear little or no resemblance to one another. In most of the above-mentioned analyses, the sequence composition of the minimal AuxRE has not been rigorously defined. In addition, whether most of these putative AuxREs are responsive to auxin as isolated elements or function only when combined with other promoter elements remains to be determined. It is also unclear whether a single type of AuxRE functions in a variety

[1] To whom correspondence should be addressed.

of different auxin-regulated genes or a number of different types of AuxREs are involved in auxin-regulated gene expression. Because auxins appear to activate a variety of genes that may function in different auxin-regulated processes (e.g., cell elongation, cell division, and cell differentiation), it is possible that a number of different types of AuxREs exist in plants. In addition, it is possible that different response elements are involved in the regulation of genes that are induced by auxins and other agents (e.g., salicylic acid, methyl jasmonate, and heavy metals) versus genes that show a strict auxin specificity (reviewed by Guilfoyle et al., 1993, Guilfoyle, 1994; T. Ulmasov, A. Ohmiya, G. Hagen, and T.J. Guilfoyle, manuscript in preparation).

Here, we report gel shift, methylation interference, deletion, site-directed mutagenesis, linker scanning, and gain-of-function analyses of the soybean *GH3* promoter. We analyzed *GH3* promoter–*GUS* reporter gene fusions in transgenic tobacco plants and in a carrot protoplast transient expression system. At least three independent AuxREs were found in the *GH3* promoter. We identified a 76-bp auxin-responsive fragment that could be further separated into two independent auxin-inducible subfragments. Two of these subfragments are only 25- and 32-bp in length and represent two of the smallest auxin-responsive fragments identified to date that can function as isolated elements. Our results indicated that the *GH3* promoter is composed of more than one functional AuxRE and that these AuxREs can function independently from one another. Furthermore, our results showed combinations of AuxREs incrementally increase the auxin inducibility of the *GH3* promoter.

RESULTS

TGA-Boxes or Hex-like Elements in the *GH3* Promoter

We have previously identified several potential regulatory sequences in the *GH3* promoter (Hagen et al., 1991; Guilfoyle et al., 1993; Guilfoyle, 1994). These include three TGA-box or Hex-like (hexamer) sequences (Tabata et al., 1989), TGACGTGGC (−235 to −227), TGACGTAA (−86 to −79), and TGACGCAG (−427 to −420), and the sequence GTCGGCGGCGCCCATTAGT (−322 to −304) resembling that found in the auxin-inducible *A. tumefaciens* gene 5 promoter and the *A. rhizogenes rolB* promoter (see Hagen et al., 1991). We have performed gel shift analysis on *GH3* promoter fragments containing the above elements with nuclear extracts prepared from wheat germ, cauliflower curd, soybean plumules, and pea plumules. Similar results have been obtained with each of these extracts.

Figure 1A shows gel shift analysis with pea nuclear extracts. The sequences TGACGTGGC and TGACGTAA represented strong binding sites for pea nuclear proteins (Figure 1A). The third Hex-like element, TGACGCAG, and the sequence related to *Agrobacterium* genes did not appear to bind nuclear proteins with high affinity (data not shown). Two major gel shift bands

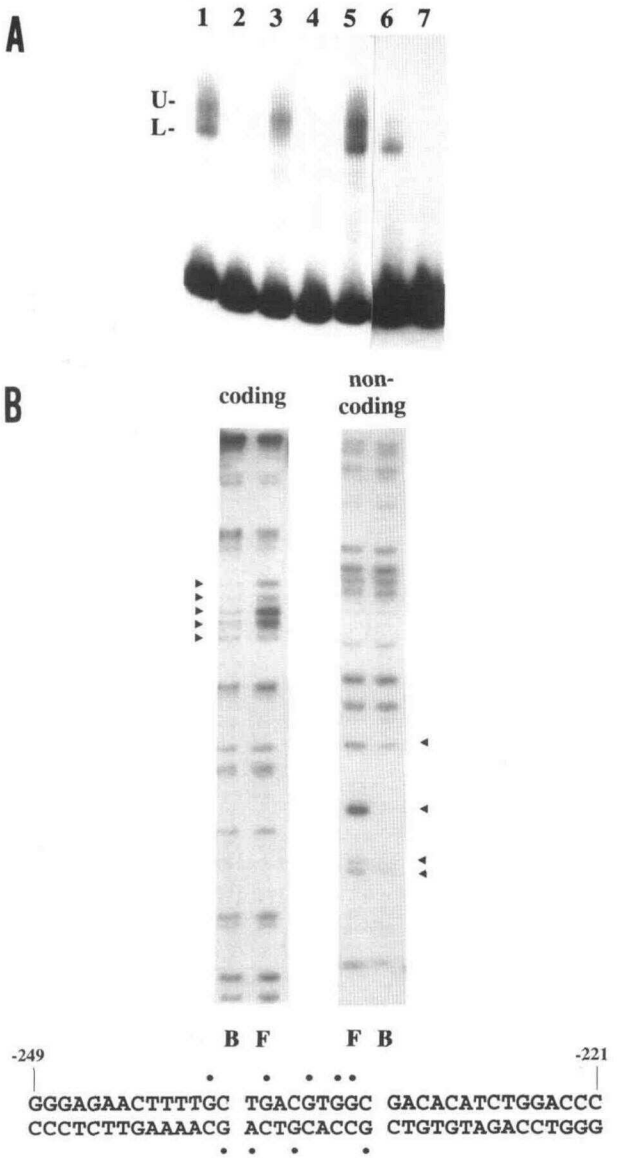

Figure 1. Two TGA-Box or Hex-like Elements in the *GH3* Promoter Are Major Binding Sites for Nuclear Proteins.

(A) Gel mobility shift analysis of nuclear proteins that bind to TGACGTGGC and TGACGTAA in the *GH3* promoter. Each lane contains 3 μg of pea nuclear protein and 1 ng of labeled probe. Probes were a 162-bp AvaI-AlwNI fragment (−266 to −105) that contains the sequence TGACGTGGC (lanes 1 to 5) and a 144-bp AflIII fragment (−209 to −66) that contains the sequence TGACGTAA (lanes 6 and 7). Double-stranded oligonucleotides were used as competitors at 200-fold molar excess. Lane 1, no competitor; lane 2, TGACGTGGC competitor; lane 3, TGACGTAA competitor; lane 4, acACGTGGC competitor; lane 5, CACCATCACCAT competitor; lane 6, no competitor; and lane 7, TGACGTAA competitor. U, upper band; L, lower band.

(B) Methylation interference with the noncoding and coding strands of a *GH3* promoter fragment containing the TGA-box or Hex-like sequence, TGACGTGGC. Pea nuclear extracts (10 μg) were incubated

were observed with a probe (−266 to −105) that contained the TGACGTGGC element, but lacked the TGACGTAA element (Figure 1A, lane 1). Several additional minor bands were observed with longer exposure times of the autoradiograms, but the DNA sequences that give rise to these minor bands have not been determined.

The oligonucleotide sequence TGACGTGGC efficiently competed with the probe for binding to both major gel shift bands (lane 2), but the oligonucleotide sequence TGACGTAA competed only for binding to the lower band (lane 3). A mutant oligonucleotide acACGTGGC also effectively competed for binding of both the upper and lower bands (lane 4). On the other hand, a nonspecific oligonucleotide probe, CACCATCACCAT, failed to compete for binding (lane 5). When a −209 to −66 fragment, which contains the TGACGTAA element but lacks the TGACGTGGC element, was used as probe, only one band was observed in gel shifts (lane 6), and the oligomer TGACGTAA competed with the probe for binding (lane 7). One binding site (the upper complex in lane 1) recognized (T/a)(G/c)ACGTGGC and resembled a G-box or Hex element in its binding properties (Katagiri and Chua, 1992). Other results with mutated sequences indicated that the terminal GGC is important for formation of the upper protein gel shift band (T. Ulmasov, unpublished results). The other binding site (the lower complex in lane 1) did not require the terminal GGC for binding because the TGACGTAA oligonucleotide was an effective competitor.

We have used methylation interference to determine that the TGACGTGGC element (−235 to −227) bound one or more nuclear proteins in pea nuclear extracts (Figure 1B). We have not carried out methylation interference on the TGACGTAA element (−86 to −79), but we have determined that this element effectively competed with the TGACGT sequence in gel shift experiments discussed above (Figure 1A).

To determine if the TGA-boxes or Hex-like sequences might be important for auxin inducibility of the *GH3* gene, we used site-directed mutagenesis to modify or destroy these binding sites in a 592-bp *GH3* promoter, and we tested the mutant promoters in transgenic tobacco plants using a *GUS* reporter gene. Figure 2A shows results with transgenic tobacco plants which indicated that mutations in TGACGTGGC (mutated to TttCGTGGC; M-1 mutation), TGACGTAA (mutated to TttCGTAA; M-3 mutation), or both sites (M-5 mutation) caused a small loss (about twofold) of auxin inducibility of the *GH3* promoter. Whereas the level of auxin inducibility decreased somewhat with these *GH3* promoter mutations when assayed as GUS

Figure 1. (continued).

with an end-labeled 162-bp AvaI-AlwNI (−266 to −105) fragment of the *GH3* promoter and treated as described in Methods. F is the free probe with no extract added, and B is bound probe after adding protein. The nucleotides, which after being methylated inhibit protein binding, are indicated with the arrows. The sequence at the bottom represents the TGA-box at −235 to −227, TGACGTGGC, and surrounding nucleotides. Closed circles show nucleotides involved in methylation interference.

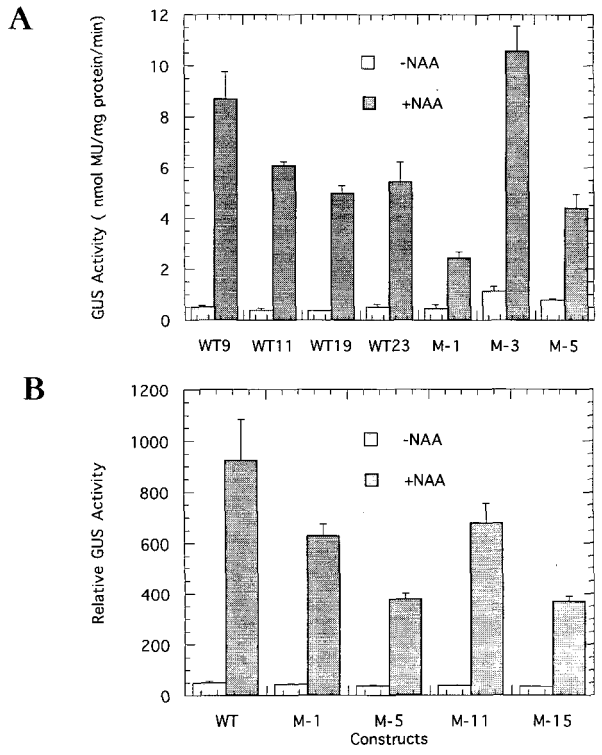

Figure 2. Quantitative Fluorometric Assays for Auxin Inducibility of the Unmutated *GH3* Promoter and TGA-Box Mutations.

(A) Independent transgenic lines of R_1 tobacco seedlings with a 592-bp wild-type (WT) *GH3* promoter (WT9, WT11, WT19, and WT23) were incubated in the presence (+) or absence (−) of 50 μM NAA for 24 hr. Mutant promoter constructs M-1 (TGACGTGGC → TttCGTGGC), M-3 (TGACGTAA → TttCGTAA), and M-5 (both M-1 and M-3 mutations in the same promoter construct) were tested by combining seedlings from four independent transgenic lines for each construct and incubating seedlings as described above. Bar graphs represent the average of four replicate assays, and standard deviations are also represented. MU, 4-methylumbelliferone.

(B) Transient assays in carrot protoplasts of M-1, M-5, M-11, and M-15 mutations in the *GH3* promoter. The M-1 and M-5 mutations are described above. The M-11 and M-15 mutations consisted of (TGACGTGGC → TttaaaGGC) and (TGACGTGGC → TttaaaGGC and TGACGTAA → TttaaaAA), respectively. Protoplasts were transfected and incubated 24 hr in the presence or absence of 50 μM NAA. Transfections were replicated four times. The average GUS activity and standard deviation are given for each construct. GUS activity is given in relative units and was standardized using luciferase as an internal standard for each transfection.

fusions in transgenic tobacco plants, the mutant promoters were still clearly auxin inducible.

We have also tested the M-1 and M-5 mutations as well as more destructive mutations M-11 (−235 to −227 TGACGTGGC mutated to TttaaaGGC) and M-15 (containing the M-11 mutation as well as the −86 to −79 TGACGTAA mutated to TttaaaAA) by using transient expression assays in carrot protoplasts. These latter mutations completely alter the ACGT core which is required for the binding of basic leucine zipper (bZIP) proteins (Katagiri and Chua, 1992). The transient expression results with M-1 and M-5 were similar to those found with transgenic tobacco plants; in these transfection assays, some reduction in the level of auxin inducibility was observed, but the mutant promoters were still auxin inducible (Figure 2B). The more destructive mutations to the TGA-box or Hex-like elements (mutations M-11 and M-15) displayed levels of auxin inducibility similar to the M-1 and M-5 mutations in carrot protoplasts. These results showed that the TGA-box or Hex-like elements may be important for the absolute level of auxin inducibility, but they must function within the context of additional independent AuxREs within the *GH3* promoter.

Deletion Analysis of the *GH3* Promoter

To gain further insight into the elements that confer auxin inducibility to the *GH3* promoter, we constructed a number of 5′ deletions, as shown in Figure 3A, and analyzed the constructs in transgenic tobacco seedlings that were either treated or not treated for 24 hr with 50 μM α-naphthalene acetic acid (NAA). Our results showed that auxin inducibility was retained in deletions up to −183, but was largely lost at −107, as shown in Figures 3B and 4. These results indicated that an AuxRE must lie between −183 and −108. The −107 and −55 deletions were not significantly inducible with auxin. The transgenic data also indicated that sequences lying between −314 and −207 increased the level of auxin inducibility of the *GH3* promoter.

For each deletion construct of the *GH3* promoter in transgenic tobacco, we examined the auxin-inducible organ-specific patterns of gene expression in seedlings and the tissue-specific patterns in excised stems and petioles. Figure 4A shows that deletions up to −314 did not alter the expression pattern of the *GUS* reporter gene compared to the full-length promoter in transgenic tobacco seedlings. A deletion to −206 appeared to result in reduction of the amount of auxin inducibility in the root and cotyledons, but we have observed at least some auxin inducibility of −206 and −183 deletions in these organs. Deletions −107 and −55 showed little or no histochemical staining for GUS activity. We have also examined the GUS staining in cross-sections of stems and petioles that were excised and incubated in the presence or absence of 50 μM NAA. The results with excised stems and petioles (Figure 4B) paralleled the relative expression levels observed with tobacco seedlings (Figure 4A) and did not reveal any obvious changes in tissue-specific gene expression in the deletion series. With the full-

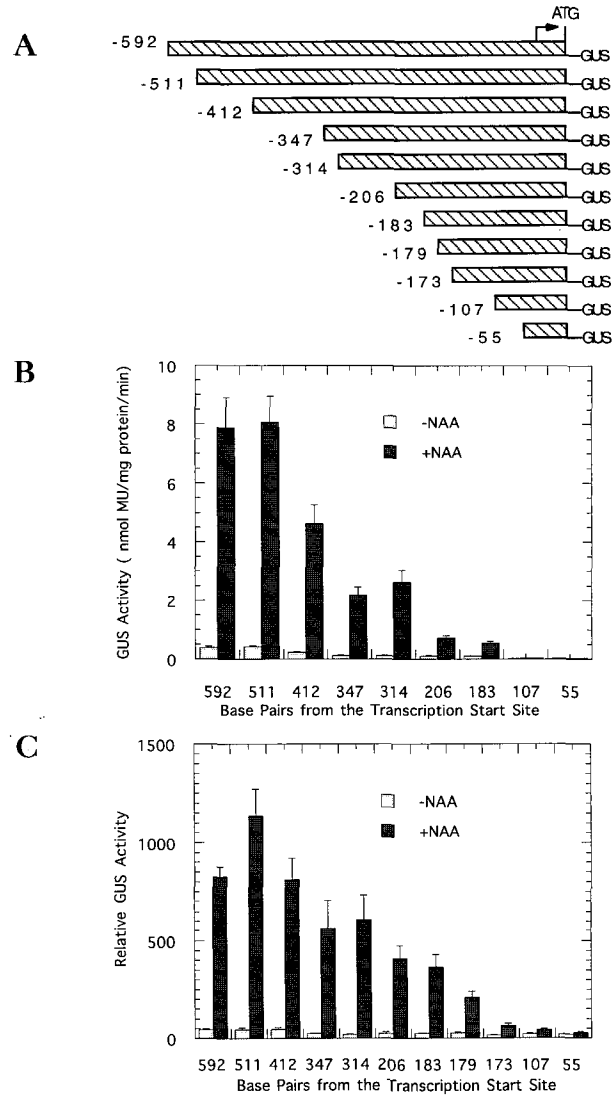

Figure 3. Quantitative Fluorometric Assays for GUS Activity with a 5′ Deletion Series of the *GH3* Promoter.

(A) Diagram of the 5′ unidirectional deletions in the *GH3* promoter. Deletion end points are indicated in base pairs from the transcription start site (indicated with an arrow). All *GH3* promoters were fused to a *GUS* reporter gene (see Methods).

(B) Gus assays in transgenic tobacco seedlings. R_1 seedlings from five to 10 independent transformants were incubated in media with (+) or without (−) 50 μM NAA for 24 hr. GUS activity was determined in tissue homogenates of each independent transgenic line. Bar graphs represent the average GUS activity and standard deviation in five to 10 independent lines for each deletion construct. MU, 4-methylumbelliferone.

(C) GUS assays in a carrot protoplast transient expression system. From five to 25 transfections with or without NAA (50 μM) were conducted for each deletion construct. The average GUS activity and standard deviation are given for each construct. GUS activity is given in relative units and was standardized using luciferase as an internal standard for each transfection.

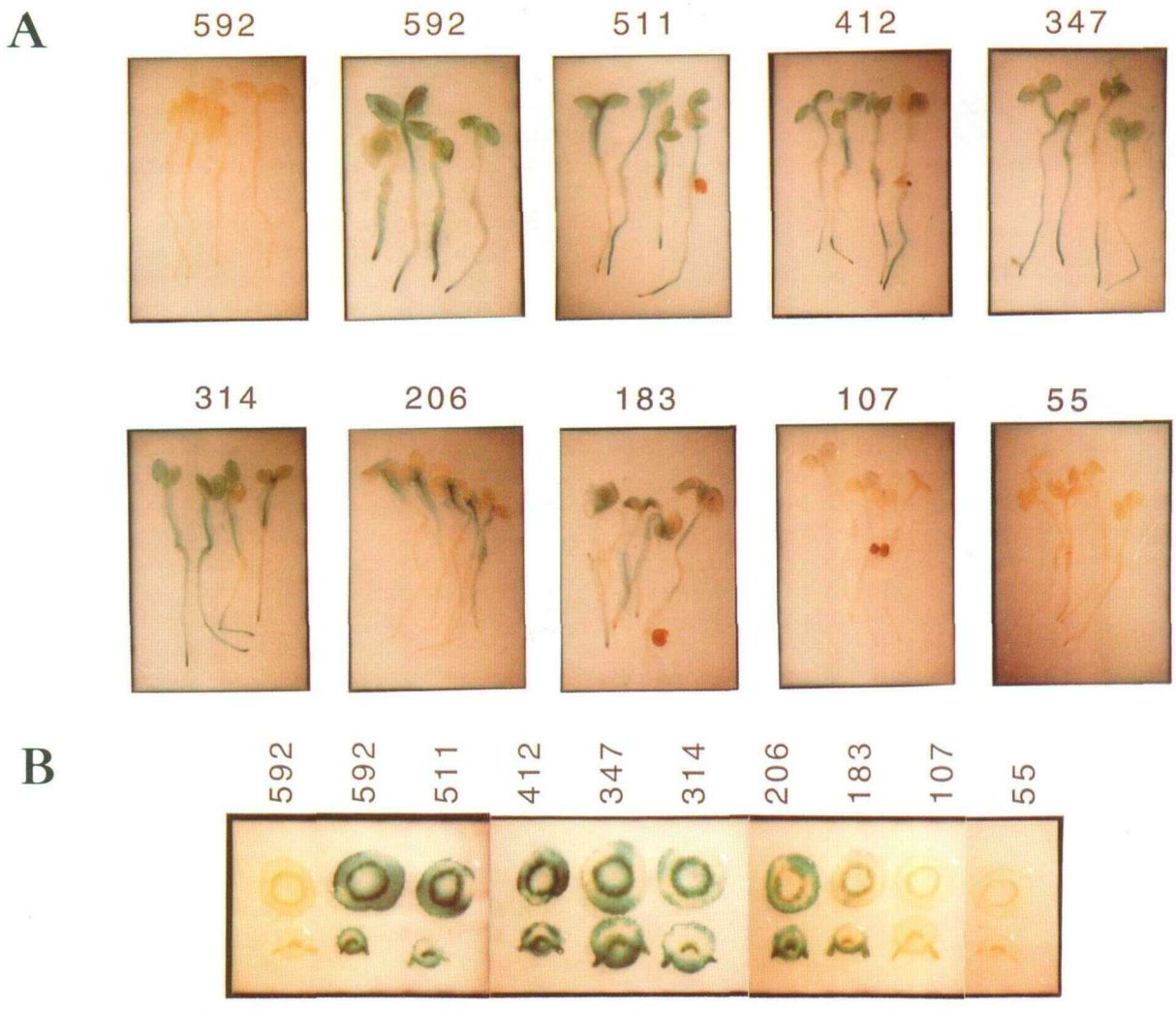

Figure 4. Histochemical Staining for GUS Activity in Transgenic R_1 Tobacco Seedlings and Excised Stems and Petioles.

Data are shown for a single representative transgenic tobacco line.

(A) Seedlings were from transformed lines containing the following *GH3* promoter deletion end points: 592, 511, 412, 347, 314, 206, 183, 107, and 55. Seedlings shown at top left (592) were incubated for 24 hr without NAA. All other seedlings shown were incubated with 50 μM NAA for 24 hr prior to histochemical staining. No GUS staining greater than that shown in seedlings at top left was detected for any of the deletion constructs incubated in the absence of NAA (data not shown).

(B) GUS staining in stem (top) and petiole (bottom) cross-sections of the same deletion series with the same NAA treatments shown in **(A)**.

length 592-bp *GH3* promoter and deletions up to −183, auxin induced GUS expression in epidermis, cortex, and vascular tissues in both excised stems and petioles. The −107 and −55 promoter deletions showed little or no histochemical staining for GUS activity.

In addition to the transgenic tobacco studies, we performed transient assays in carrot protoplasts with the deletion series of the *GH3* promoter. The transient assays confirmed that sequences lying between −183 to −108 contained an AuxRE (Figure 3C). In contrast to the transgenic analysis, we observed a small amount of auxin induction (twofold with a standard error of 0.5) with the −107 deletion. Whether the low level of induction with the −107 deletion in transient assays is significant has not been clarified. Another difference from the transgenic studies was that transient assays did not show such a large enhancement in auxin inducibility with sequences upstream of −206 (i.e., −314 to −592). The reason for the difference in the effect of sequences upstream of −206 in transgenic versus transient assays is not clear at this time, but could be related to tissue-specific expression that is not retained in cultured carrot cells. It is also possible that the differences observed in transgenic and transient assays resulted from the

failure of the DNA to package into a normal chromatin structure and/or a high copy number of DNA templates in transient assays. Because results with transient assays in carrot protoplasts were qualitatively similar to those obtained with transgenic tobacco plants and because transient assays required much less time for analysis, the transient assay system was used to further analyze the *GH3* promoter.

Linker Scanning between −183 and −108 of the *GH3* Promoter

To further delimit the AuxRE that is localized between −183 to −108, we conducted linker scanning throughout this auxin-inducible *GH3* promoter fragment. The −183 to −108 linker scanning mutations were conducted within a 592-bp *GH3* promoter fused to the *GUS* reporter gene. In this set of experiments, the wild-type 592-bp *GH3* promoter was induced by auxin about 13-fold in carrot protoplast transient assays, as shown in Figure 5. Most of the linker scanning mutations between −183 to −108 had no significant effect on the level of auxin inducibility of the *GH3* promoter. Only mutations L1 (−183 to −174) and L5 (−143 to −134) altered the auxin inducibility. The L1 mutation caused about a 2.5-fold decrease in the level of induction by auxin (i.e., induced about fivefold by auxin), and this resulted primarily from the higher level of expression in the absence of auxin addition compared to other mutations and the wild-type promoter. The L5 mutation caused about a twofold increase in auxin inducibility (i.e., induced 20-fold by auxin), which resulted primarily from a lower level of expression in protoplasts incubated in the absence of auxin.

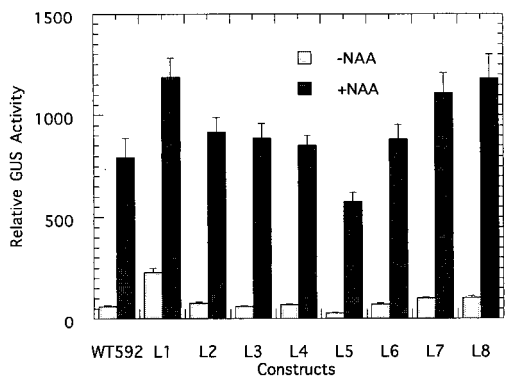

Figure 5. Linker Scanning Mutation Analysis of the −183 to −108 *GH3* Promoter Fragment in Carrot Protoplasts.

A BglII linker sequence, gaagatcttc, was used to replace 10-bp segments from −183 to −124, and a BglII linker sequence, aagatctt, was used to replace an 8-bp segment from −123 to −108 in the 592-bp *GH3* promoter. The following linker scanning mutations were tested: L1, −183 to −174; L2, −173 to −164; L3, −163 to −154; L4, −153 to −144; L5, −143 to −134; L6, −133 to −124; L7, −123 to −116; and L8, −115 to −108. Each linker scan construct was assayed 10 to 30 times by transient assay in carrot protoplasts incubated in the presence or absence of 50 μM NAA for 24 hr. Relative GUS activity was determined using luciferase as an internal standard. WT592, wild-type 592-bp *GH3* promoter.

These results indicated that although some small perturbations in the auxin inducibility of the *GH3* promoter were observed in the linker scanning mutations with the −183 to −108 auxin-inducible fragment, the promoter was still induced about fivefold by auxin even with the most destructive mutation, L1. Our failure to clearly identify an AuxRE between −183 and −108 could have resulted from the presence of more than one AuxRE within the 592-bp *GH3* promoter. To determine if this was the case, we conducted gain-of-function analysis with fragments of the *GH3* promoter.

Auxin-Inducible Fragments in Addition to the −183 to −108 Fragment in the *GH3* Promoter

To determine if an AuxRE existed outside of the −183 to −108 fragment of the *GH3* promoter, we fused a number of different promoter fragments to a −46 CaMV 35S promoter–*GUS* reporter gene, as shown in Figure 6A, and tested these constructs for auxin inducibility in carrot protoplasts. We observed an enhanced level of auxin induction when sequences upstream of −183 were included in a fragment extending to −110 at its 3′ end. Figure 6B shows that the −243 to −110 and −229 to −110 constructs gave a slightly higher level of auxin inducibility (about 12-fold to 15-fold) compared to a −183 to −108 construct (about eightfold). The amount of induction by auxin with the −243 to −110 and −229 to −110 was close to the 13- to 18-fold induction observed with the 592-bp *GH3* promoter in carrot protoplast transient assays (see Figure 3C, column 592, and Figure 5, column WT592). It is unlikely that the TGA-box, TGACGTGGC, (−235 to −227) contributes to the auxin inducibility of the −243 to −110 fragment because the −229 to −110 fragment was induced to about the same level by auxin as the −243 to −110 fragment.

Figure 6B also shows that a *GH3* promoter (originating from the 592-bp promoter) with an internal deletion from −180 to −110 (Δ[−180–110]) was induced about sixfold by NAA. Furthermore, a *GH3* promoter fragment, −249 to −203, could function as an isolated auxin-inducible fragment when fused as a multimer to the minimal CaMV 35S promoter. These results indicated that at least two independent auxin-inducible fragments exist in this promoter: one upstream of −183 (between −249 and −203) and one between −183 and −108. We have not determined which sequences are important for auxin inducibility in the *GH3* promoter upstream of −183; however, we have found that the sequence (−322 to −304) related to the sequence within the *A. tumefaciens* gene 5 promoter is not auxin inducible as an isolated element fused to a minimal −46 CaMV 35S promoter (Figure 6B). We have also observed that a −94 to −74 construct that contained the Hex-like element, TGACGTAA, was not auxin inducible (Figure 6B), even though a −107 deletion construct showed a low level of auxin inducibility in transient assays (see Figure 3C).

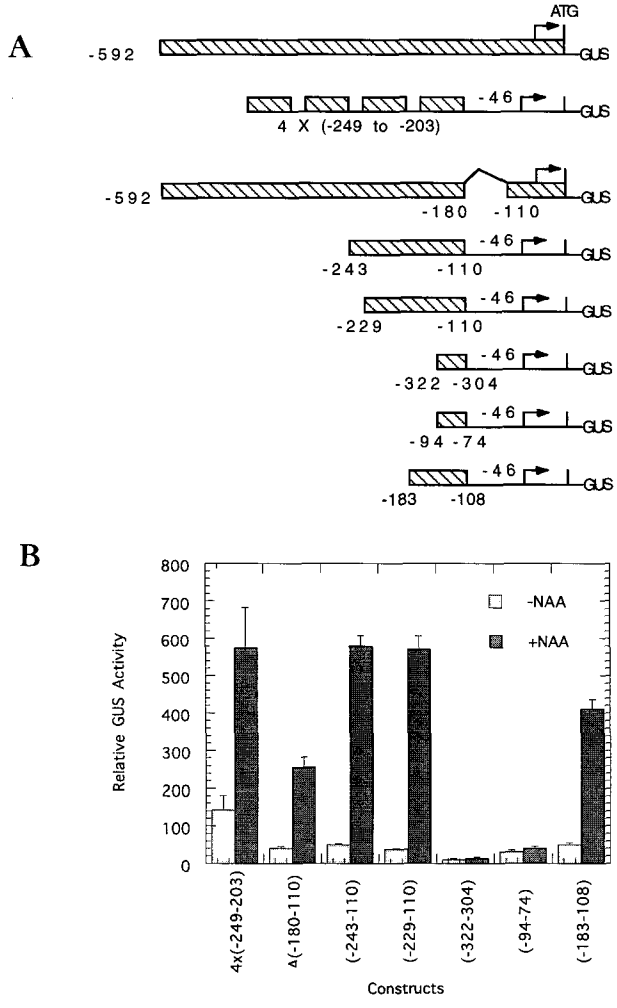

Figure 6. A Promoter Fragment Upstream of the −183 to −107 Fragment in the *GH3* Promoter Is Auxin Inducible.

(A) Diagram of the promoter fragments tested by transient assays in carrot protoplasts. End points of fragments tested are indicated. The construct at the top represents the 592-bp wild-type *GH3* promoter. Promoter fragments 4×(−249 to −203), (−243 to −110), (−229 to −110), (−322 to −304), (−94 to −74), and (−183 to −108) were fused to the minimal −46 CaMV 35S promoter. An internal deletion of the 592-bp *GH3* promoter from −180 to −110 (Δ[−180–110]) was also tested by transient assay. The 4×(−249 to −203) construct was composed of four tandem copies of a promoter fragment (−249 to −203) that were all in the correct orientation. The remainder of the constructs contained only a single copy of each fragment in the correct orientation.

(B) Quantitative GUS assays in a carrot protoplast transient expression system. Carrot protoplasts were incubated in the presence or absence of 50 μM NAA for 24 hr prior to assaying for GUS activity. Each construct was tested five to 10 times with and without auxin addition to the protoplasts. The average GUS activity and standard deviation are given for each construct. GUS activity is given in relative units and was standardized using luciferase as an internal standard for each transfection.

Gain-of-Function Analysis with the −183 to −108 *GH3* Promoter Fragment

To determine the sequences responsible for auxin inducibility within the −183 to −108 *GH3* promoter fragment, we conducted gain-of-function analysis with this promoter fragment and subfragments. We fused the −183 to −108 fragment and subfragments to a minimal −46 CaMV 35S promoter–*GUS* reporter gene, and these constructs were tested by transient assays in carrot protoplasts. The −183 to −108 construct (D0) gave about eightfold induction with auxin in carrot protoplasts, as shown in Figure 7; this compared favorably with the 13-fold induction observed with the 592-bp *GH3* promoter. Figure 8 diagrams the *GH3* promoter. When this larger fragment was broken into two smaller fragments, −183 to −159 (D1) and −158 to −108 (D3), and these were fused to a minimal CaMV 35S promoter, both of these smaller fragments were induced approximately threefold with auxin. The −158 to −108 fragment was further divided into two pieces, −158 to −135 (D2) and −142 to −111 (D4). Whereas the −158 to −135 fragment showed no significant auxin inducibility, the −142 to −111 fragment was induced approximately threefold by auxin. Multimerization (four or five copies in the correct orientation) of both the D1 (5×D1), −183 to −159, and the D4 (4×D4), −142 to −111, fragments increased the level of auxin inducibility to approximately fivefold and sevenfold, respectively. These results demonstrated that an auxin-inducible −183 to −108

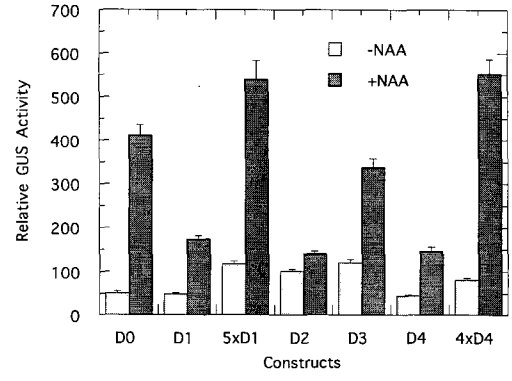

Figure 7. Gain-of-Function Analysis for a Minimal CaMV 35S Promoter Containing Subfragments of the −183 to −108 *GH3* Promoter Fragment.

GH3 promoter subfragments fused to a minimal −46 CaMV 35S promoter were tested in a carrot protoplast transient assay system. The following *GH3* promoter fragments were tested: D0, −183 to −108; D1, −183 to −159; 5×D1, five copies (in the correct orientation) of D1; D2, −158 to −135; D3, −158 to −108; D4, −142 to −111; and 4×D4, four copies (in the correct orientation) of D4. See Figure 8 for a diagram of these fragments in the *GH3* promoter. Relative GUS activity was determined using luciferase as an internal standard. Transfections and assays were replicated from five to 15 times for each construct. Protoplasts were incubated in the presence and absence of 50 μM NAA for 24 hr prior to GUS assays.

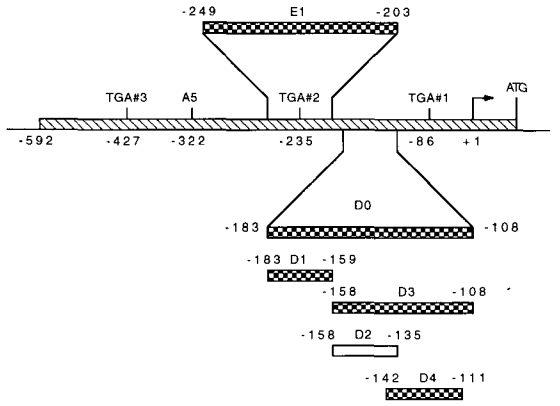

Figure 8. Diagram of the *GH3* Promoter.

(A) A diagram of the 592-bp *GH3* promoter and auxin-inducible subfragments of this promoter. The transcription start site is indicated with an arrow, and the translation start site is indicated with an ATG. TGA#1 (TGACGTAA) and TGA#2 (TGACGTGGC) are two major sites that bind proteins in nuclear extracts from pea, soybean, wheat germ, and cauliflower. TGA#3 (TGACGCAG) does not bind proteins in these extracts. A5 indicates the 5′ position of the sequence (GTCGGCGGCGCCCATTAGT) that is related to a sequence found in the *A. tumefaciens* T-DNA gene 5 (see Hagen et al., 1991). Fragments of the promoter found to be auxin inducible when fused to a minimal CaMV 35S promoter are indicated by the checkered boxes. The open box (D2), located within D0, is a fragment that is not auxin inducible.

(B) DNA sequences contained in the D1, D3, D4, and E1 fragments of the *GH3* promoter. Boldface letters indicate the conserved sequence elements within the AuxREs between −183 to −108.

fragment within the *GH3* promoter is composed of two independent auxin-inducible subfragments, each consisting of ~25 to 32 bp in length. Each of these subfragments contained a conserved sequence element consisting of TGTCTCctcAATAAG (−176 to −162) and TGTCTC---AATAAG (−130 to −119).

DISCUSSION

The major findings of this study are summarized below. First, a 592-bp *GH3* promoter conferred auxin inducibility to a *GUS* reporter gene assayed by transient expression in carrot protoplasts. This *GH3* promoter–*GUS* fusion was previously shown to be auxin inducible as a stable construct in transgenic tobacco (Hagen et al., 1991). Second, two elements, TGACGTGGC and TGACGTAA, which are predicted to bind bZIP proteins, were identified as strong binding sites in the *GH3* promoter with nuclear extracts prepared from four different plant species (results with pea nuclear extracts were shown). When these sites were mutated, the *GH3* promoter retained its auxin inducibility. Third, promoter deletion analysis indicated that the auxin inducibility was still maintained in a promoter deleted to −183, but was completely (in stable transformants) or almost completely (in transient assays) lost in a promoter deleted to −107. This indicated that a major auxin-inducible element in the *GH3* promoter resided in a 76-bp fragment between −183 and −108. Fourth, this 76-bp fragment conferred auxin inducibility to a minimal −46 CaMV 35S promoter. This indicated that the 76-bp fragment contained one or more AuxREs that can act independently from the remainder of the *GH3* promoter. Fifth, the 76-bp fragment was not the only auxin-inducible fragment within the *GH3* promoter because sequences upstream of −183 enhanced the auxin inducibility. When a promoter fragment from −180 to −110 was deleted from the 592-bp *GH3* promoter, the promoter with this internal deletion was still induced sixfold by auxin. Furthermore, a fragment from −249 to −203 was auxin inducible when fused as a multimer to a minimal CaMV 35S promoter. Sixth, the 76-bp fragment (−183 to −108) can be split into two subfragments, each about 25 to 32 bp in length, and each subfragment retained about half of the amount of auxin inducibility as the entire 76-bp fragment. This indicated that the 76-bp fragment contained at least two independent AuxREs. Seventh, the 25- and 32-bp subfragments derived from the larger 76-bp fragment each contained a conserved element, TGTCTCctcAATAAG (−176 to −162) and TGTCTC---AATAAG (−130 to −119).

In total, our results indicated that the *GH3* promoter is composed of a complex array of AuxREs, all of which appear to contribute to the overall magnitude of auxin induction displayed by this promoter. A summary diagram of the auxin-inducible fragments within the *GH3* promoter is presented in Figure 8. At least three of these AuxREs can function independently of one another, and when brought together, they incrementally increase the level of auxin induction of the *GH3* promoter.

We have conducted a detailed analysis of the 76-bp auxin-inducible *GH3* promoter fragment, and this has led to the identification of a conserved sequence element that appears to be crucial for auxin inducibility. Part of the conserved element within each 25- and 32-bp AuxRE of the *GH3* promoter, TGTCTC, is also found in the NDE promoter region of the auxin-inducible small auxin-up RNA (*SAUR*) genes (McClure et al., 1989). These *SAUR* promoter regions that contain the TGTCTC sequence element are known to be required for the auxin inducibility of the *SAUR* genes (Y. Li, Z.-B. Liu, X. Shi, G. Hagen, and T.J. Guilfoyle, manuscript submitted for publication). In addition, a number of other auxin-inducible genes contain similar, if not identical, elements within their promoters (see Guilfoyle, 1994); however, it has not been determined whether these sequences function as AuxREs in other auxin-inducible genes. It is unlikely that the TGTCTC element can function as an AuxRE in isolation, because we have found that this

element, when fused to a minimal −46 CaMV 35S promoter, has no basal activity and is not auxin inducible (T. Ulmasov and Z.-B. Liu, unpublished results). On the other hand, we have found that the TGTCTC element is required for auxin inducibility of both 25- and 32-bp AuxREs in the *GH3* promoter (T. Ulmasov and Z.-B. Liu, unpublished results).

Functional analysis of several auxin-inducible promoters has led to the identification of a number of other putative AuxREs. An et al. (1990) reported that an element, GCACATACGT, within the nopaline synthase (*nos*) promoter was both wound- and auxin-inducible. This element contains an ACGT core common to a number of elements that bind bZIP proteins (Katagiri and Chua, 1992). A similar core sequence (actually tandem sequences) occurs in the octopine synthase (*ocs*) and *ocs*-like elements, which have also been shown to be auxin inducible (Liu and Lam, 1994). Our recent results indicate that the *ocs*-like element in the auxin-inducible soybean *GH2/4* promoter (see Hagen et al., 1984, 1988; Ellis et al., 1993) is not specifically induced by auxins, but is induced by a variety of agents including nonauxin analogs, salicylic acid, and abscisic acid (T. Ulmasov, A. Ohmiya, G. Hagen, and T.J. Guilfoyle, manuscript in preparation). Kim et al. (1994) have recently reported that *nos*, *ocs*, and CaMV 35S *as-1* elements, which contain similar hexamer motifs, confer auxin, methyl jasmonate, and salicylic acid inducibility to a *nos* promoter. Based on these latter results, it is unclear whether *ocs*-like elements are simply induced by chemical and environmental stresses or are a separate class of AuxREs that display less than strict auxin specificity. Nagao et al. (1993) have identified Hex-like elements in the auxin-inducible *GmAux28* promoter that bind proteins in nuclear extracts prepared from soybean. A number of other sequences in this auxin-inducible promoter that bind nuclear proteins were also identified. It remains to be established, however, whether these protein binding sites are involved in the auxin inducibility of the *GmAux28* gene. Our results indicated that the TGA-box or Hex-like elements may play some role in the regulation of the *GH3* promoter but did not allow us to determine if these elements function as independent AuxREs in the *GH3* promoter.

Korber et al. (1991) mapped auxin-responsive sequences to a 90-bp region within the promoter of the *A. tumefaciens* auxin-responsive T-DNA gene 5 of the pAch5 Ti plasmid. A fragment of this 90-bp region, GTCGGCGGCGggtCCCATTtGT, showed an 18 of 21 nucleotide match with a sequence in the *GH3* promoter (−322 to −304), GTCGGCGGCG—CCCATTaGT (Hagen et al., 1991). Ballas et al. (1993) recently reported that a 164-bp fragment (−318 to −154) is required for auxin inducibility of the pea indoleacetic acid–inducible gene *PS-IAA4/5*. Whether one or more smaller fragments can function as an AuxRE when excised from the remainder of the 164-bp promoter has not been demonstrated. Based upon similarity to sequences in other auxin-responsive genes, they proposed that the AuxRE within the *PS-IAA4/5* gene consisted of the sequence TGTCCCAT (−187 to −180 in the pea promoter) with a consensus sequence of T/GGTCCCAT. Their deletion analysis indicates, however, that this element is not auxin inducible by itself, because a −188 deletion of the *PS-IAA4/5* promoter has little or no basal activity and does not respond to auxin (Ballas et al., 1993).

The putative AuxRE described in pea is identical to a part of the auxin-inducible region in the T-DNA gene 5 promoter described above, and a seven-of-nine nucleotide match is found in a part of the −322 to −304 sequence in the *GH3* promoter. Our results presented here indicate, however, that this sequence (−322 to −304) is not auxin inducible in a transient assay system (see Figure 5). On the other hand, this entire −322 to −304 element or the GCGCCCAT sequence contained in the −322 to −304 element might contribute to the enhanced auxin inducibility observed with *GH3* promoter deletions up to −314 (compared to the −206 and −183 deletions; see Figure 3). In any case, some sequence(s) between −314 to −205 enhances the auxin inducibility of the *GH3* promoter in transgenic tobacco plants.

The most 5′ portion of *PS-IAA4/5* putative AuxRE (Ballas et al., 1993) is similar to the TGTCTC element found in both 25- and 32-bp auxin-inducible fragments within the *GH3* promoter. Interestingly, the NDE promoter region in soybean *SAUR* genes (McClure et al., 1989) contains both a *PS-IAA4/5* element, T/GGTCCCAT, and a *GH3* element, TGTCTC, within a few base pairs of one another. It may be that both of these elements function either independently or cooperatively with one another in regulating the auxin inducibility of the *SAUR* genes. It remains to be determined whether the T/GGTCCCAT element identified in the pea *PS-IAA4/5* and the TGTCTC element in the soybean *GH3* promoter play similar or identical roles in conferring auxin inducibility to a promoter. The conserved nature of the 5′ ends of these putative AuxREs suggests that these elements might be related to one another.

We have determined that the internal TC within the TGTCTC element is critical for auxin inducibility, whereas the TG and TC at the ends of the element can be mutated without loss of auxin inducibility (T. Ulmasov, unpublished results). The internal TC within the *GH3* element (third and fourth nucleotides) is conserved in the *PS-IAA4/5* element (third and fourth nucleotides). In addition to the possible relationship between the TGTCTC and T/GGTCCCAT elements, we have observed that a sequence just 3′ to the putative AuxRE in the *PS-IAA4/5* promoter consists of TGTCaCCcCtATAAG (−176 to −162; Ballas et al., 1993); this sequence is similar to the sequence TGTCTCCTCAATAAG (−176 to −162) within the 25-bp AuxRE in the *GH3* promoter. It should now be possible to determine how the TGTCTC element functions within the context of other sequences in the 25- and 32-bp auxin-inducible fragments and to clarify whether the pea *PS-IAA4/5* TGTCCCAT or the TGTCACCCCTATAAG and the soybean *GH3* TGTCTC or TGTCTCCTCAATAAG elements play similar roles in auxin-inducible promoters.

Our results indicated that the *GH3* promoter is much more complex in its composition of AuxREs compared to previously published analyses of auxin-inducible promoters (An et al.,

1990; Korber et al., 1991; Ballas et al., 1993; Kim et al., 1994; Liu and Lam, 1994). Each auxin-inducible fragment within the *GH3* promoter appears to contribute incrementally to the overall auxin inducibility of this promoter. Whereas the *GH3* promoter appears to be more complex in its number of auxin-inducible elements compared to the pea *PSIAA4/5* (Ballas et al., 1993) and the soybean *SAUR 15A* (Y. Li, Z.-B. Liu, X. Shi, G. Hagen, and T.J. Guilfoyle, manuscript submitted for publication) promoters, it is possible these promoters also contain multiple auxin-inducible elements. One or more of the element(s) in the *PSIAA4/5* and *SAUR* promoters could function similarly to those in the *GH3* promoter. There are, however, at least two major differences between the expression patterns displayed by the *GH3* promoter and the *PSIAA4/5* and *SAUR* promoters; the *PSIAA4/5* and *SAUR* promoters show a much higher level of expression in the absence of exogenous auxin and a more restricted pattern of organ- and tissue-specific expression than the *GH3* promoter (Theologis et al., 1985; McClure and Guilfoyle, 1987; Gee et al., 1991; Hagen et al., 1991; Li et al., 1991; Ballas et al., 1993). The multiplicity and/or organization of AuxREs in the *GH3* promoter may contribute to these differences in expression patterns.

METHODS

Plant Material

Transgenic tobacco (*Nicotiana tabacum* cv Xanthi-nc) seedlings (R_1 generation) containing the *GH3* promoter, promoter deletions, or promoter mutations were germinated and grown on moistened sand at 20°C under fluorescent lights. Five- to 7-day-old tobacco seedlings were removed from the sand bed, rinsed with distilled H_2O, and incubated with shaking for 24 hr at 22°C. The incubation medium consisted of 10 mM potassium phosphate buffer, pH 7.0, and 50 μg/mL chloramphenicol plus or minus 50 μM α-naphthalene acetic acid (NAA). Cross-sections (1-mm-thick) of transgenic tobacco petioles or stems were excised and incubated as described for tobacco seedlings. Carrot (*Daucus carota*) suspension culture cells were obtained from Wendy Boss, North Carolina State University (Raleigh) and grown at 20°C on a rotatory shaker (150 rpm) in Murashige and Skoog (1962) media (Sigma) containing 2 mg/L NAA and 0.4 mg/L kinetin. Cultures were maintained by aliquoting cultured cells and adding 9 volumes of fresh media every 7 days.

Protoplast Isolation

Carrot protoplasts were isolated from log phase suspension-cultured cells using a modification of the procedure of Goodall et al. (1990). After 3 to 4 days of subculture, 50 mL of carrot cells were sedimented at 200*g* for 2 min in a Beckman GSA13.1 rotor and resuspended in 25 mL of a solution containing 5 mM 2-(*N*-morpholino)ethane-sulfonic acid, pH 5.7, 0.4 M sorbitol, and 2% (w/v) Driselase (Sigma). The cells were transferred to a Petri dish and gently shaken at 20°C for 4 to 5 hr. Light microscopy was used to monitor the time course of protoplast preparation. The protoplasts were pelleted at 60*g* for 10 min in a GSA13.1 rotor, and the supernatant was removed by aspiration. The protoplasts were gently suspended in 30 mL (per 50-mL culture) of W5 solution (Goodall et al., 1990) and counted using a hemocytometer and light microscopy. From a 50-mL culture, the yield was $\sim 10^8$ protoplasts. The protoplasts were repelleted and suspended in W5 solution at 2×10^6 protoplasts per mL.

Protoplast Transfection and GUS Assays

Freshly prepared carrot protoplasts in W5 solution, isolated as described above, were pelleted and resuspended in MC buffer (5 mM 2-(*N*-morpholino)ethane-sulfonic acid, pH 5.7, 20 mM $CaCl_2$, and 0.5 M mannitol) at 2×10^6 protoplasts per mL. For transfection assays, 6×10^5 protoplasts in 0.3-mL MC solution were mixed at room temperature with 10 μL of supercoiled plasmid DNA constructs in Tris-EDTA buffer. The DNA constructs consisted of a mixture of 5 μg of a soybean *GH3* promoter–β-glucuronidase (*GUS*) reporter gene plasmid and 0.1 μg of a 941-bp 35S cauliflower mosaic virus (CaMV) promoter–firefly luciferase reporter gene (Barnes, 1990; provided by John Rogers, Washington University, St. Louis). The luciferase construct served as an internal standard for each transfection assay. We have observed no auxin inducibility with the 941-bp 35S CaMV promoter–luciferase construct in carrot protoplast transient assays. The protoplasts and plasmid DNAs were mixed with 0.3 mL of 40% (w/v) polyethylene glycol containing 0.1 mM $Ca(NO_3)_2$ and 0.4 M mannitol, pH 10, at room temperature for 5 min. Four milliliters of Murashige and Skoog media, pH 5.7, with or without auxin, was added, and protoplasts were incubated in the dark at room temperature for 24 hr. For each *GH3* promoter construct, 10 transfection assays were conducted with the same protoplast preparation. Five of these assays contained no auxin, and five assays contained 5×10^{-5} M NAA.

Following incubation, the protoplasts were pelleted by centrifugation at 60*g* for 15 min. The protoplasts were lysed in 200 μL of luciferase cell culture lysis reagent (Promega), and luciferase activity was determined with a luciferase assay system according to the supplier's instructions (Promega). For the luciferase assay, 2 μL of cell lysate was added to 100 μL of Promega's luciferase assay reagent; the mixture was immediately placed into a luminometer (Analytical Scientific Instruments, Alameda, CA), and the emitted photons were counted for 10 sec. Values for luciferase activity were generally within the range of 50,000 to 500,000 photons per 10 sec. Lysates from cells that were not transfected with the luciferase reporter gene had values of ~200 photons per 10 sec. A second 2 μL of cell lysate was used to determine GUS activity using 1.7 mM of the fluorescent 4-methylumbelliferyl β-D-glucuronide as substrate, as described by Jefferson (1987). To standardize GUS activity for the various promoter constructs and transfections, GUS activity for each transfection was divided by the luciferase activity for that same transfection. The average GUS activity and standard error were calculated using five or more replications for each auxin-induced and uninduced transfected plasmid construct.

Construction of Promoter Deletions, Plant Transformation, and GUS Assays

The 592-bp *GH3* promoter–*GUS* reporter gene construct previously described by Hagen et al. (1991) was used as the full-length wild-type *GH3* promoter construct. This construct was digested with ClaI, and a series of 5′ unidirectional deletions in the *GH3* promoter were made using Promega's Erase-a-Base system according to the manufacturer's instructions. Ten deletions of the 592-bp *GH3* promoter were

constructed: 511, 412, 347, 314, 206, 183, 179, 171, 107, and 55 bp from the transcription start site. The unidirectional *GH3* promoter deletion–*GUS* reporter gene constructs were transformed into and propagated in *Escherichia coli* DH5αF′. The plasmid constructs were isolated by equilibrium centrifugation on CsCl gradients containing ethidium bromide (Ausubel et al., 1987). Each deletion construct was sequenced at its 5′ end to determine the deletion end point. The *GH3* promoter deletion–*GUS* reporter gene inserts were excised from the plasmid constructs by digestion with EcoRI/partial HindIII, resolved in and isolated from agarose gels, and ligated to pMON505 (Horsch et al., 1985; Horsch and Klee, 1986; Rogers et al., 1987). The pMON505 constructs were mobilized into the binary vector pTi37-SE of *Agrobacterium* using the triparental mating method and were used to transform *N. tabacum* cv Xanthi-nc by the leaf disc methods described by Horsch et al. (1985) and Rogers et al. (1987).

Regenerated R_0 plantlets were transferred to soil and grown to maturity. Five to 10 independent transgenic R_0 plants were self pollinated for seed production, and the R_1 progeny were used for *GH3* promoter–*GUS* reporter gene analysis. Quantitative fluorometric and histochemical GUS assays were performed as described by Jefferson (1987) and Hagen et al. (1991) using 5- to 7-day-old light-grown R_1 seedlings or cross-sections of stem and petioles from fully expanded leaves that were incubated with shaking in 10 mM potassium phosphate buffer or buffer plus 5×10^{-5} M NAA for 24 hr. After histochemical staining, seedlings, stem cross-sections, and petiole cross-sections were cleared with a graded series of ethanol concentrations.

Construction of *GH3* Promoter Mutations in the TGA-Boxes

To obtain mutant variants of the *GH3* promoter that modified or destroyed the TGA-boxes or Hex-like sequences (Hagen et al., 1991), we used a novel polymerase chain reaction (PCR)–based mutagenesis procedure, the details of which will be published elsewhere (T. Ulmasov and T.J. Guilfoyle, manuscript in preparation). Two types of mutations were constructed for both the distal (−235 to −227) and proximal (−86 to −79) TGA-boxes or Hex-like elements (Hagen et al., 1991; Katagiri and Chua, 1992) in the *GH3* promoter. Mutation M-1 converts TGACGTGGC (−235 to −227) to TttCGTGGC, and mutation M-3 converts TGACGTAA (−86 to −79) to TttCGTAA in the *GH3* 592-bp promoter. Mutation M-5 contains both mutations M-1 and M-3 in the *GH3* promoter. A second set of mutations were made that completely destroyed the ACGT core in the Hex-like elements. Mutation M-11 converts the −235 to −227 element to TttaaaGGC, and mutation M-13 converts the −86 to −79 element to TttaaaAA. Mutation M-15 contains both mutation M-11 and M-13 in the 592-bp *GH3* promoter. Mutant constructs M-1, M-3, and M-5 were fused to the *GUS* reporter gene, cloned into the binary vector pMON505 (Horsch et al., 1985), transformed into *A. tumefaciens* by electroporation (Shen and Forde, 1989), and transformed into tobacco as described above. The auxin inducibility of the mutant M-1, M-3, and M-5 constructs was tested in R_1 transgenic seedlings. Mutant constructs M-1, M-5, M-11, and M-15 were also examined for auxin inducibility in a carrot protoplast transient assay system described above.

Linker Scanning

A 10-bp BglII linker, gaagatcttc, was used to replace 10-bp increments from −183 to −124 of the 592-bp *GH3* promoter. An 8-bp BglII linker, aagatctt, was used to replace 8-bp increments from −123 to −108 of the promoter. These linker scanning mutations were made using a PCR-based method described by Gustin and Burk (1993). Eight linker scanning mutations were constructed and these are referred to as L1 (−183 to −174), L2 (−173 to −164), L3 (−163 to −154), L4 (−153 to −144), L5 (−143 to −134), L6 (−133 to −124), L7 (−123 to −116), and L8 (−115 to −108). Each construct was sequenced using Sequenase (U.S. Biochemical Corp.) to confirm the sequences of the mutant constructs.

Construction of Chimeric *GH3*/−46 CaMV Promoters

A number of *GH3* promoter fragments between −183 to −108, upstream of −183 and downstream of −110, were fused to a −46 CaMV 35S promoter with a tobacco mosaic virus 5′ leader (derived from pAGUS1; Skuzeski et al., 1990) to test for regions of the *GH3* promoter that confer auxin inducibility. Oligonucleotide primers and PCR were used to make the constructs, and each construct was confirmed by sequencing. All manipulations were conducted using standard protocols described by Ausubel et al. (1987).

To make constructs containing only 25 and 32 nucleotide fragments between −183 and −111 of the *GH3* promoter, the following oligonucleotides were synthesized:

oligonucleotide 1: ggggccctcgtgtctcctcaataagcta, sense strand from −183 to −159;
oligonucleotide 2: ccctagcttattgaggagacacgagggc, nonsense strand from −183 to −159;
oligonucleotide 3: gggcacaatttgaaacatacacgcaat, sense strand from −158 to −135;
oligonucleotide 4: cccattgcgtgtatgtttcaaattgtg, nonsense strand from −158 to −135;
oligonucleotide 5: cacgcaatcctttgtctcaataagttccactc, sense strand from −142 to −111;
oligonucleotide 6: gtgagtggaacttattgagacaaaggattgc, nonsense strand from −142 to −111.

Oligonucleotides 1 and 2, 3 and 4, and 5 and 6 were mixed in equal quantities (4 μg per oligonucleotide), heated to 90°C for 5 min, and annealed by slowly cooling to 18°C. Incubation was continued at 18°C for 12 hr. Nucleotide extensions were added to the 5′ ends of the oligonucleotides to allow formation and ligation of multimers using T4 DNA ligase. Ligation mixtures, containing both monomers and oligomers, were made blunt ended with mung bean nuclease and fused to the −46 CaMV 35S promoter as described above. Chimeric promoters containing either one, four, or five copies of *GH3* promoter fragments −183 to −159, −158 to −135, and −142 to −111 were cloned and confirmed by sequencing.

Preparation of Nuclear Extracts, Gel Mobility Shift, and Methylation Interference Analyses

Nuclei were isolated from pea plumules as described by Hagen and Guilfoyle (1985). Prior to isolation of nuclei, pea plumules (50 g per treatment) were incubated for 1 hr in buffer containing 10 mM potassium phosphate, pH 6.0, 2% (w/v) sucrose, and 50 μg/mL chloramphenicol or buffer plus 5×10^{-5} M 2,4-dichlorophenoxyacetic acid. Purified nuclei were suspended in 5 mL of nuclear extract buffer consisting of 20 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl, 20% (v/v) glycerol, 100 μg/mL phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. To this,

ammonium sulfate was added to a concentration of 0.4 M, and the solution was stirred gently for 30 min and cleared by centrifugation for 1 hr at 40,000 rpm in a Beckman Ti75 rotor. The supernatant was brought to 70% saturation with ammonium sulfate and gently stirred for 30 min; nuclear protein was pelleted by centrifugation (40,000 rpm for 1 hr). The pellet was suspended in 0.4 mL of nuclear extract buffer containing 60 mM KCl but no $MgCl_2$, and the suspension was dialyzed against the same buffer for 6 hr. Precipitated protein was removed by centrifugation in a refrigerated microcentrifuge, and the supernatants were aliquoted, frozen in liquid N_2, and stored at −80°C.

Gel shift assays were performed as described by Ausubel et al. (1987) and Singh et al. (1986). Probes consisted of phosphorus-32 end-labeled promoter fragments. These included 162-bp AvaI-AlwNI (−266 to −105) and 144-bp AflIII (−209 to −66) *GH3* promoter fragments. Approximately 1 ng of end-labeled probe and 2 to 6 μg of nuclear extract were used for each binding reaction along with 2 μg of poly(dI-dC) as nonspecific competitor.

A 162-bp AvaI-AlwNI fragment of the *GH3* promoter containing the distal TGA-box or Hex-like element, TGACGTGGC, (−236 to −228) was used in methylation interference experiments according to protocols described by Ausubel et al. (1987).

ACKNOWLEDGMENTS

This research was supported by National Science Foundation Grant Nos. DCB 8904493 and IBN 9303956. This is Paper No. 12,066 of the Journal Series of the Missouri Agricultural Experimental Station. We thank John Rogers for the luciferase reporter gene construct.

Received January 31, 1994; accepted March 28, 1994.

REFERENCES

Ainley, W.M., Walker, J.C., Nagao, R.T., and Key, J.L. (1988). Sequence and characterization of two auxin-regulated genes from soybean. J. Biol. Chem. **263,** 10658–10666.

Alliotte, T., Tiré, C., Engler, G., Peleman, J., Caplan, A., Van Montagu, M., and Inzé, D. (1989). An auxin-regulated gene of *Arabidopsis thaliana* encodes a DNA-binding protein. Plant Physiol. **89,** 743–752.

An, G., Costa, M.A., and Ha, S.-B. (1990). Nopaline synthase promoter is wound inducible and auxin inducible. Plant Cell **2,** 225–233.

Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1987). Current Protocols in Molecular Biology, Vol. 2. (New York: John Wiley and Sons).

Ballas, N., Wong, L.-M., and Theologis, A. (1993). Identification of the auxin-responsive element, *AuxRE*, in the primary indoleacetic acid-inducible gene, *PS-IAA4/5*, of pea (*Pisum sativum*). J. Mol. Biol. **233,** 580–596.

Barnes, W. (1990). Variable patterns of expression of luciferase in transgenic tobacco leaves. Proc. Natl. Acad. Sci. USA **87,** 9183–9187.

Conner, T.W., Goekjian, V.H., LaFayette, P.R., and Key, J.L. (1990). Structure and expression of two auxin-inducible genes from *Arabidopsis*. Plant Mol. Biol. **15,** 623–632.

Dominov, J.A., Stenzler, L., Lee, S., Schwarz, J.J., Leisner, S., and Howell, S.H. (1992). Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell **4,** 451–461.

Ellis, J.G., Tokuhisa, J.G., Llewellyn, D.J., Bouchez, D., Singh, K., Dennis, E.S., and Peacock, W.S. (1993). Does the *ocs*-element occur as a functional component of the promoters of plant genes? Plant J. **4,** 433–443.

Gee, M.A., Hagen, G., and Guilfoyle, T.J. (1991). Tissue-specific and organ-specific expression of soybean auxin-responsive transcripts GH3 and SAURs. Plant Cell **3,** 419–430.

Goodall, G., Wiebauer, K., and Filipowicz, W. (1990). Analysis of pre-mRNA processing in transfected plant protoplasts. Methods Enzymol. **181,** 148–155.

Guilfoyle, T.J. (1994). Auxin-regulated genes and promoters. In Biochemistry and Molecular Biology of Plant Hormones, K.R. Libbenga and M.A. Hall, eds (Dordrecht: Elsevier Publishing Company), in press.

Guilfoyle, T.J., Hagen, G., Li, Y., Ulmasov, T., Liu, Z., Strabala, T., and Gee, M. (1993). Auxin-regulated transcription. Aust. J. Plant Physiol. **20,** 489–502.

Gustin, K.E., and Burk, R.D. (1993). A rapid method for generating linker scanning mutants utilizing PCR. Biotechniques **14,** 22–23.

Hagen, G., and Guilfoyle, T.J. (1985). Rapid induction of selective transcription by auxin. Mol. Cell. Biol. **5,** 1197–1203.

Hagen, G., Kleinschmidt, A.J., and Guilfoyle, T.J. (1984). Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta **162,** 147–153.

Hagen, G., Uhrhammer, N., and Guilfoyle, T.J. (1988). Regulation of expression of auxin-induced soybean sequence by cadmium. J. Biol. Chem. **263,** 6442–6446.

Hagen, G., Martin, G., Li, Y., and Guilfoyle, T.J. (1991). Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. Plant Mol. Biol. **17,** 567–579.

Horsch, R.B., and Klee, H.J. (1986). Rapid assay of foreign gene expression in leaf discs transformed by *Agrobacterium tumefaciens*: Role of T-DNA borders in the transfer process. Proc. Natl. Acad. Sci. USA **83,** 4428–4432.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Wallroth, M., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Ishida, S., Takahashi, Y., and Nagata, T. (1993). Isolation of cDNA of an auxin-regulated gene encoding a G protein β subunit-like protein from tobacco BY-2 cells. Proc. Natl. Acad. Sci. USA **90,** 11152–11156.

Jefferson, R.A. (1987). Assay for chimeric genes in plants: The GUS fusion system, Plant Mol. Biol. Rep. **5,** 387–405.

Katagiri, F., and Chua, N.-H. (1992). Plant transcription factors: Present knowledge and future challenges. Trends Genet. **8,** 22–27.

Kim, Y., Buckley, K., Costa, M.A., and An, G. (1994). A 20 nucleotide upstream element is essential for the nopaline synthase (*nos*) promoter activity. Plant Mol. Biol. **24,** 105–117.

Korber, H., Strizhov, N., Staiger, D., Feldwisch, J., Olsson, O., Sanberg, G., Palme, K., Schell, J., and Koncz, C. (1991). T-DNA gene 5 of *Agrobacterium* modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants. EMBO J. **10,** 3983–3991.

Li, Y., Hagen, G., and Guilfoyle, T.J. (1991). An auxin-responsive promoter is differentially induced by auxin gradients during tropisms. Plant Cell **3,** 1167–1175.

Liu, X., and Lam, E. (1994). Two binding sites for the plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. J. Biol. Chem. **269,** 668–675.

Maurel, C., Brevet, J., Barbier-Brygoo, H., Guern, J., and Tempe, J. (1990). Auxin regulates the promoter of the root-inducing *rolB* gene of *Agrobacterium rhizogenes* in transgenic tobacco. Mol. Gen. Genet. **223,** 58–64.

McClure, B.A., and Guilfoyle, T.J. (1987). Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Mol. Biol. **9,** 611–623.

McClure, B.A., Hagen, G., Brown, C.S., Gee, M.A., and Guilfoyle, T.J. (1989). Transcription, organization, and sequence of an auxin-regulated gene cluster in soybean. Plant Cell **1,** 229–239.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 493–497.

Nagao, R.T., Goekjian, V.H., Hong, J.C., and Key, J.L. (1993). Identification of protein binding DNA sequences in an auxin-regulated gene of soybean. Plant Mol. Biol. **21,** 1147–1162.

Oeller, P.W., Keller, J.A., Parks, J.E., Silbert, J.E., and Theologis, A. (1993). Structural characterization of the early indoleacetic acid-inducible genes, *PS-IAA4/5* and *PS-IAA6*, of pea (*Pisum sativum*). J. Mol. Biol. **233,** 789–798.

Rogers, S.G., Klee, J.H., Horsch, R.B., and Fraley, R.T. (1987). Improved vectors for plant transformation: Expression cassette vectors and new selectable markers. Methods Enzymol. **153,** 252–277.

Shen, W.J., and Forde, G.G. (1989). Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucl. Acids Res. **17,** 8385.

Singh, H., Sen, R., Baltimore, D., and Sharp, P.A. (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobin genes. Nature **319,** 154–158.

Skuzeski, J.M., Nichols, L.M., and Gesteland, R.F. (1990). Analysis of leaky viral translation termination codons in vivo by transient expression of improved β-glucuronidase vectors. Plant Mol. Biol. **15,** 65–69.

Tabata, T., Takase, H., Takayama, S., Mikami, K., Nakatsuka, A., Kawata, T., Nakayama, T., and Iwabuchi, M. (1989). A protein that binds to a *cis*-acting element of wheat histone genes has a leucine zipper motif. Science **245,** 965–967.

Takahashi, Y., Kuroda, H., Tanaka, T., Machida, Y., Takebe, I., and Nagata, T. (1989). Isolation of an auxin-regulated gene cDNA expressed during the transition from G_0 to S phase in tobacco mesophyll protoplasts. Proc. Natl. Acad. Sci. USA **86,** 9279–9283.

Takahashi, Y., Kusaba, M., Hiraoka, Y., and Nagata, T. (1991). Characterization of the auxin-regulated *par* gene from tobacco mesophyll protoplasts. Plant J. **1,** 327–332.

Theologis, A., Huynh, T.V., and Davis, R.W. (1985). Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. J. Mol. Biol. **183,** 53–68.

van der Zaal, E.J., Droog, F.N.J., Boot, C.J.M., Hensgens, L.A.M., Hoge, J.H.C., Schilperoort, R.A., and Libbenga, K.R. (1991). Promoters of auxin induced genes from tobacco can lead to auxin-inducible and root tip-specfic expression. Plant Mol. Biol. **16,** 983–998.

Walker, J.C., and Key, J.L. (1982). Isolation of cloned cDNAs to auxin-responsive poly(A)$^+$ RNAs of elongating soybean hypocotyl. Proc. Natl. Acad. Sci. USA **79,** 7185–7189.

Yamamoto, K.T., Mori, H., and Imaseki, H. (1992a). Novel mRNA sequences induced by indole-3-acetic acid in sections of elongating hypocotyls of mung bean (*Vigna radiata*). Plant Cell Physiol. **33,** 13–20.

Yamamoto, K.T., Mori, H., and Imaseki, H. (1992b). cDNA cloning of indole-3-acetic acid-regulated genes: Aux22 and SAUR from mung bean (*Vigna radiata*) hypocotyl tissue. Plant Cell Physiol. **33,** 93–97.